

Using an Enzymatic Combinatorial Approach to Identify Anticoagulant Heparan Sulfate Structures

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SUMMARY

Heparan sulfate (HS) represents a major class of glycans that perform central physiological functions. Emerging HS and glycosaminoglycan microarray techniques are used to interrogate the structure and function relationship to develop novel therapeutic agents. Availability of HS with specific sulfation patterns has been a limiting factor and impedes the accuracy of HS glycomics studies. Although organic synthesis provides oligosaccharides, these may not fully represent the biological functions of polysaccharides. Here, we present a study for developing an enzyme-based approach to synthesize a polysaccharide library with different sulfation patterns. Using different combinations of biosynthetic enzymes, we synthesized eight unique polysaccharides. We discovered that polysaccharides without the iduronic acid residue displayed strong binding affinity to antithrombin and high anti-Xa and anti-IIa activities. The enzyme-based synthetic approach could become a general method for discovering new HS structures with unique biological functions.

INTRODUCTION

Heparin, a commonly used anticoagulant drug, is a special form of heparan sulfate (HS). HS or heparin contains glucuronic/iduronic acid and glucosamine carrying sulfo groups. A large body of evidence has demonstrated that this highly sulfated polysaccharide plays a role in regulating embryonic development and inflammatory response and assisting viral/bacterial infections and blood coagulation [1]. The wide range of biological functions of HS attracts considerable interest in exploiting heparin or heparin-like molecules for the development of anticancer and antiviral drugs in addition to using them for anticoagulant purposes [2, 3]. The uniquely distributed sulfation pattern of the HS polysaccharide is believed to regulate its functional specificity [4–6]. An approach for synthesizing

a polysaccharide with specific sulfation patterns will aid in deciphering the structure and function of HS for the development of HS-based drugs [7].

Chemical synthesis has been the major route by which to obtain structurally defined heparin and HS oligosaccharides [8]. The most important example is the synthesis of the antithrombin-binding pentasaccharide. Currently, a synthetic anticoagulant pentasaccharide has been marketed worldwide under the trade name Arixtra. However, chemical synthesis of nonanalog heparin and HS oligosaccharides, larger than hexasaccharides, is extremely difficult, if not impossible, based on currently available methods for carbohydrate synthesis. While a number of groups continue to pursue the synthesis of heparin [9–11], it has become clear that chemical synthesis with current technology alone will be incapable of generating most larger oligosaccharide structures. The availability of an *in vitro* system by which to synthesize HS polysaccharides by using biosynthetic enzymes offers a promising alternative approach [12, 13].

The biosynthesis of HS involves a series of specialized sulfotransferases and other enzymes. Control of the sulfation pattern mainly depends on the substrate specificities of the enzymes involved in the biosynthetic pathway. HS is initially synthesized as a linear copolymer of glucuronic acid (GlcUA) and *N*-acetylated glucosamine (GlcNAc), which then undergoes various modifications. These modifications are carried out by a series of biosynthetic enzymes, including glucosaminyl *N*-deacetylase/*N*-sulfotransferase, which converts the GlcNAc unit to an *N*-sulfo glucosamine (GlcNS) unit (Figure 1A). After the *N*-sulfation, C₅-epimerase converts the GlcUA unit to an iduronic acid (IdoUA) unit. The resultant polysaccharide is further modified by 2-*O*-sulfotransferase (2-OST), 6-*O*-sulfotransferase (6-OST), and 3-*O*-sulfotransferase (3-OST) to introduce the 2-*O*-sulfo group to IdoUA/GlcUA and the 6-*O*-sulfo/3-*O*-sulfo groups to the glucosamine unit, respectively (Figure 1A) [14]. 6-OST is present in three isoforms, and 3-OST is present in seven isoforms.

In this article, we reported an enzyme-based combinatorial approach to synthesize the polysaccharides with different sulfation patterns (Figure 1B). From the products, we discovered that polysaccharides without the IdoUA residue displayed strong binding affinity to antithrombin and high anti-Xa and anti-IIa activities. Further, these

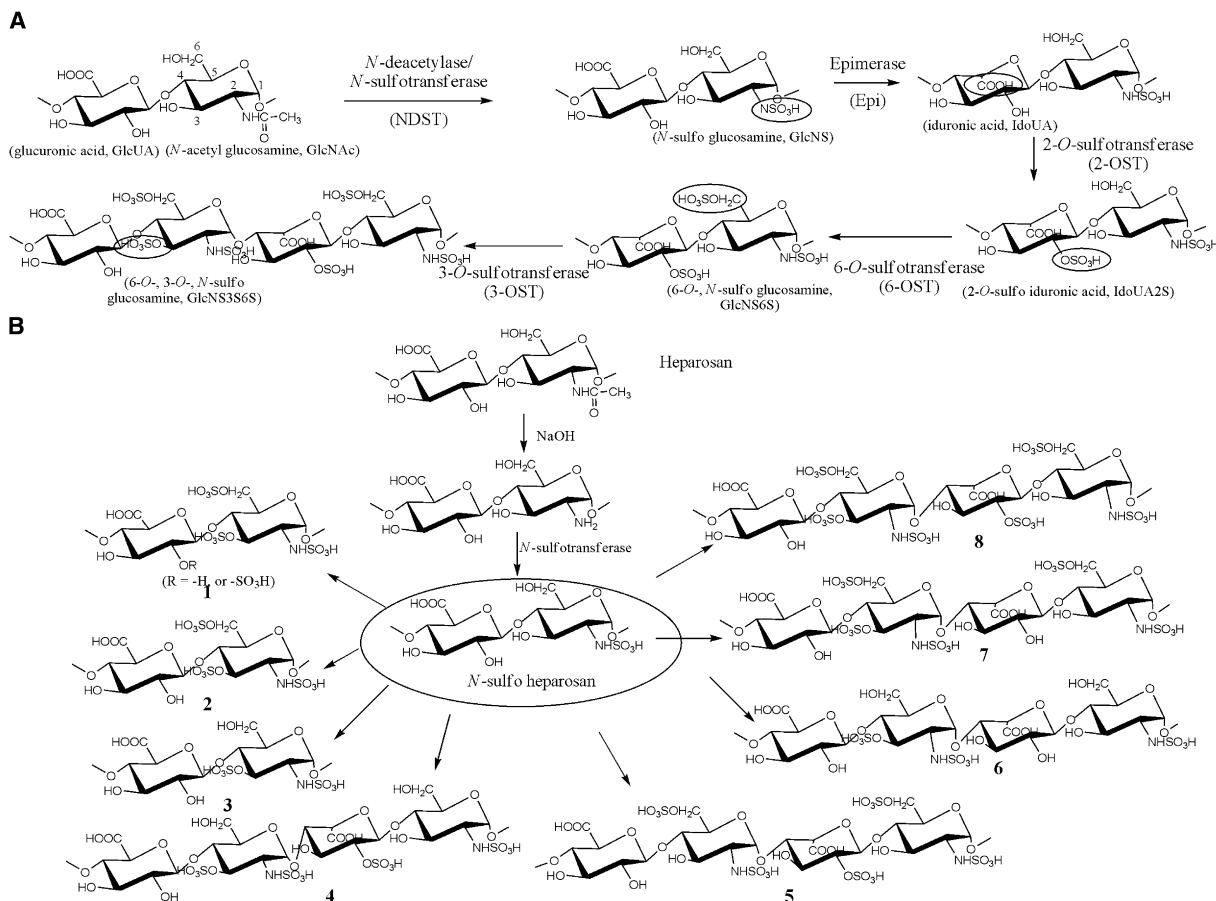


Figure 1. Biosynthetic Pathway of HS and the Synthetic Scheme of Polysaccharide Variants

(A) The reactions catalyzed by HS biosynthetic enzymes. The reaction sites at each modification step are circled. The names and abbreviations for the saccharide units are displayed underneath the sugar unit.

(B) The steps involved in the synthesis of a library of HS-like polysaccharides. The enzymes used for each compound are shown in Table 1.

polysaccharides have no activity in promoting cell proliferation, thus providing evidence for the synthesis of a functionally specific anticoagulant polysaccharide. Chemical synthesis of the IdOUA residue takes ten steps [15]. Although the synthesis of IdOUA can be achieved by epimerase, the product contains a mixture of IdOUA and GlcUA because the reaction catalyzed by epimerase is reversible [16]. Consequently, the product has greater structural heterogeneity, increasing the untoward effects of an anticoagulant drug. Anticoagulant polysaccharide without the IdOUA residues could decrease the structural heterogeneity and reduce the complexity in the synthesis of HS-based anticoagulant drugs. Because HS is also involved in tumor growth and viral infections, the enzyme-based synthetic approach could be used to prepare the HS structures displaying anticancer and antiviral activities.

RESULTS AND DISCUSSION

In an effort to establish the synthesis of a HS polysaccharide library, we have expressed essentially all HS biosynthetic enzymes in *Escherichia coli* in large quantities [4, 7].

We started the synthesis from a capsular polysaccharide of the *E. coli* K5 strain, known as heparosan, which is like a nonsulfated and unepimerized HS (Figure 1). In addition, heparosan can be converted into anticoagulant HS by using HS biosynthetic enzymes [12, 17]. Because *N*-sulfation is critical for the subsequent *O*-sulfations and epimerization [18], we decided to prepare polysaccharides with different types of *O*-sulfation patterns by using *N*-sulfo heparosan as a starting material (Figure 1B). Eight different polysaccharides that differ by the type of sulfations, such as 2-*O*-, 3-*O*-, and 6-*O*-sulfations, as well as by the level of IdOUA residues, as illustrated in Figure 1B, were prepared. The overall sulfation level among the products was between 0.9 and 1.4 nmoles *O*-sulfo groups per microgram of polysaccharide, namely, 0.39–0.61 *O*-sulfo groups per disaccharide unit, as measured based on the specific ³⁵S radioactivity (Table 1). Compound 6 was an exception because it contained only 3-*O*-sulfation, which is an inherently low-abundance sulfation type in the HS isolated from a natural source [19]. Subjecting the synthetic polysaccharides to an antithrombin (AT)-affinity column, we found that a significant portion of compounds 1,

Table 1. Summary of the Synthetic Polysaccharides and the Results of Their Bindings to Antithrombin

Compounds	Modification Enzymes	Structural Features ^a	O- ³⁵ S-Labeled Sulfation Level (nmol / μ g Polysaccharide)	Binding to Antithrombin Affinity Column (%) ^b	Binding Constant to Antithrombin (K _D) (nM) ^c
1	2-OST, 6-OST-1, 6-OST-3, and 3-OST-1	No iduronic acid residue	1.4	51%	27 nM
2	6-OST-1, 6-OST-3, and 3-OST-1	No iduronic acid residue; no 2-O-sulfation	1.1	52%	57 nM
3	3-OST-1 and 2-OST	No iduronic acid residue; no 6-O-sulfation	0.9	1.0%	Not determined
4	Epi, 2-OST, and 3-OST-1	No 6-O-sulfation	1.3	0.7%	Not determined
5	Epi, 2-OST, 6-OST-1, and 6-OST-3	No 3-O-sulfation	1.5	0.6%	Not determined
6	Epi and 3-OST-1	No 2-O- and 6-O-sulfations	0.4	5.1%	Not determined
7	Epi, 6-OST-1, 6-OST-3, and 3-OST-1	No 2-O-sulfation	1.3	29%	25 nM
8	Epi, 6-OST-1, 6-OST-3, 3-OST-1, and 2-OST	Very similar to the HS isolated from tissues	1.5	35%	29 nM

Epi, C₅-epimerase.

^aThese structural features are compared to heparin and HS isolated from natural sources.

^bThe percentage of the ³⁵S-labeled polysaccharide bound to the AT-affinity column was determined by incubating AT and the polysaccharides. The complex of AT and polysaccharide was captured by using a ConA-agarose column.

^cThe binding affinity of the polysaccharides and AT was determined by using affinity coelectrophoresis.

2, **7**, and **8** bound to AT, while **3**, **4**, **5**, and **6** showed much less or no binding to AT (Table 1). We noted that **1**, **2**, **7** and **8** all carry 3-O- and 6-O-sulfo groups, while **3**, **4**, **5**, and **6** either lacked 3-O-sulfo or 6-O-sulfo groups. Thus, we concluded that both 3-O-sulfation and 6-O-sulfation of the glucosamine unit are critical for binding to AT. In addition, 2-O-sulfation of IdoUA is not essential, provided that **7** binds to AT. These conclusions are largely consistent with previously published results of chemically synthesized pentasaccharides [20] as well as enzymatically synthesized polysaccharides [12, 21]. The binding constants (K_Ds) of the synthetic polysaccharides and AT were determined to be in the range of 27–57 nM (Table 1), which are very similar to that of previously characterized anticoagulant HS [17].

The synthetic polysaccharides displayed excellent anti-coagulant activity, as measured by anti-Xa and anti-IIa activities (Table 2). The IC₅₀ values of compounds **1**, **2**, **7**, and **8** for Xa are 2- to 2.5-fold higher than that of unfractionated heparin, while they are about 2-fold lower than that of Lovenox, a low-molecular weight heparin drug manufactured by Aventis. A similar trend was observed for the anti-IIa activity of these compounds. The *N*-sulfo heparosan 6-O-sulfate was used as a negative control and showed no inhibitory effect on the activity of Xa and IIa.

Among the synthesized AT-binding polysaccharides, compound **2** has the simplest structure because it should contain neither IdoUA nor 2-O-sulfo groups. To further elucidate the structure of the synthesized polysaccharides, we conducted the disaccharide analysis of compound **2** and compared it to the disaccharide composition

of compound **8**. The resultant disaccharides were resolved on reverse-phase ion pairing (RPIP) HPLC (Figure 2). Indeed, six disaccharides were observed in compound **8**, while only three disaccharides were observed in compound **2**. In addition, we noted that the ratio of the three disaccharides in **2**, including GlcUA-AnMan3S, GlcUA-AnMan6S, and GlcUA-AnMan3S6S, is about 1:2:1 (Table 3), suggesting that this polysaccharide consists of a repeating tetrasaccharide unit with a structure of -GlcUA-GlcNS6S-GlcUA-GlcNS3S \pm 6S-. The results from disaccharide analysis also concluded that the IdoUA residue is not important for the polysaccharide to bind to AT.

Table 2. Anti-Xa and Anti-IIa Activities of the Synthetic Polysaccharides

Compounds	Anti-Xa Activity, IC ₅₀ (ng/ml)	Anti-IIa Activity, IC ₅₀ (ng/ml)
Heparin	25	8
1	50	18
2	50	20
7	45	16
8	40	15
<i>N</i> -sulfo heparosan 6-O-sulfate ^a	>5000	>3000
Lovenox ^b	90	50

^aThe synthesis of *N*-sulfo heparosan 6-O-sulfate is displayed in Figure S2.

^bLovenox was purchased from a local pharmacy.

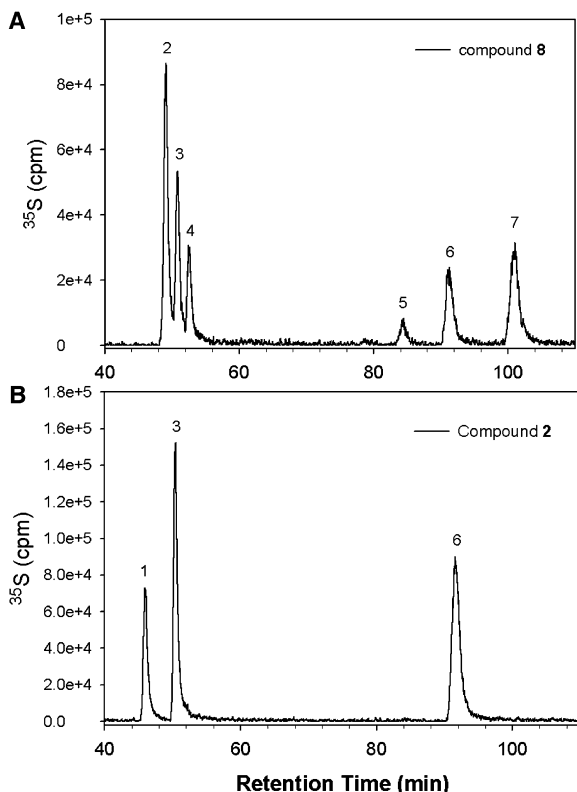


Figure 2. RP/HP-HPLC Chromatograms of the Disaccharide Analysis of Compounds 8 and 2

(A and B) The ^{35}S -labeled polysaccharides were degraded by nitrous acid at pH 1.5, followed by sodium borohydride reduction. The resultant ^{35}S -labeled disaccharides were desalted on a BioGel P-2 column, and then resolved by reverse-phase ion pairing HPLC chromatography. The elution positions were identified by coeluting with ^{35}S -labeled or ^3H -labeled disaccharide standards; 1 represents GlcUA-AnMan3S, 2 represents IdoUA2S-AnMan, 3 represents GlcUA-AnMan6S, 4 represents IdoUA-AnMan6S, 5 represents IdoUA-AnMan3S6S, 6 represents GlcUA-AnMan3S6S, and 7 represents IdoUA2S-AnMan6S.

The fact that compound **2** exhibits high binding affinity to AT and strong anticoagulant activity appeared to be contradictory to previously published results. The IdoUA residue has been implicated to be essential for the binding to AT because it provides conformational flexibility [22]. In the pentasaccharides, the IdoUA residue exists in either a chair ($^1\text{C}_4$) or a skew boat ($^2\text{S}_0$) conformation [22]. Furthermore, the unique skew boat ($^2\text{S}_0$) of IdoUA2S in a synthetic pentasaccharide was proved to be essential for the binding to AT [23]. We believe that the degree to which conformational flexibility is necessary for AT-binding affinity is size dependent. To test this hypothesis, we compared the AT-binding oligosaccharides' synthetic efficiency by using a mixture of enzymes with or without the capability of synthesizing IdoUA2S. In one preparation, the oligosaccharides (for the synthesis of oligosaccharides without the IdoUA2S residue) were incubated with a mixture of enzymes containing 6-OST-1, 6-OST-3, and 3-OST-1. In another preparation, the oligosaccha-

Table 3. Compositional Analysis of Compounds 2 and 8

Disaccharides	Compound 2 (mol/mol %)	Compound 8 (mol/mol %)
GlcUA-AnMan3S	24.7%	N.D. ^a
IdoUA2S-AnMan	N.D.	35.9%
GlcUA-AnMan6S	48.8%	23.3%
IdoUA-AnMan6S	N.D.	15.9%
IdoUA-AnMan3S6S	N.D.	2.2%
GlcUA-AnMan3S6S	26.2%	8.9%
IdoUA2S-AnMan6S	N.D.	13.4%

^a Not detectable.

rides (for the synthesis of oligosaccharides with the IdoUA2S residue) were incubated with a mixture of enzymes containing epimerase and 2-OST as well as 6-OST-1, 6-OST-3, and 3-OST-1. The oligosaccharide substrates with structures of $\Delta\text{UA}-(\text{GlcNS-GlcUA})_n\text{-GlcNS-}$, where $n = 0, 1, 2, 3, 4$, and ≥ 5 , were prepared by subjecting *N*-sulfo heparosan to partial depolymerization with heparin lyase III, followed by fractionation on gel permeation chromatography (Figure S1; see the Supplemental Data available with this article online). After the modifications, the AT-binding, ^{35}S -labeled oligosaccharides were captured by the AT-affinity column, and the data are shown in Figure 3. In the absence of epimerase and 2-OST, ^{35}S -labeled octa- and decasaccharides showed substantial binding to AT, while ^{35}S -labeled hexasaccharide showed a very low yield. In contrast, when using the same oligosaccharide substrates in the presence of epimerase and 2-OST, ^{35}S -labeled hexasaccharides displayed significant binding to AT. We noted that the amount of AT-binding hexasaccharides and octasaccharides is about 4- to 5-fold higher than when the substrates were modified with a mixture of enzymes containing epimerase and 2-OST. These results suggest that the contribution of the IdoUA2S to the AT-binding affinity may be less essential when the size of the oligosaccharides is larger than an octasaccharide.

Heparin and HS form a ternary complex with fibroblast growth factors (FGFs) and fibroblast growth factor receptors (FGFRs) to stimulate cell proliferation; however, this interaction clearly is not related to the anticoagulant activity of HS. Here, we tested whether the synthetic polysaccharides permit the separation of the anticoagulant activity and cell proliferation activity by using a BaF3 cell model system. The BaF3 cells overexpressing FGFR1 normally depend on IL-3 for growth. In the absence of IL-3, cell proliferation depends on the addition of both FGF and heparin or HS [24]. The cells receiving heparin and compound **8** showed an increase in [^3H]thymidine incorporation (Figure 4). Compound **8** stimulated BaF3 cell growth to a lesser extent than heparin since its structure is closer to HS than heparin [7]. We also found that compound **1** showed modest activity in stimulating cell proliferation. Compounds **2** and **7** had no detectable activity in stimulating cell

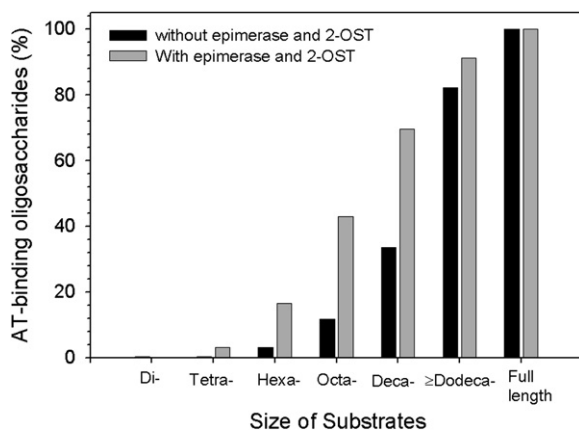


Figure 3. Enzymatic Synthesis of AT-Binding Oligosaccharides

The oligosaccharide substrates (1 μ g) or the full-length *N*-sulfo heparosan (1 μ g) were incubated with a mixture of enzymes, including 6-OST-1 (20 μ g), 6-OST-3 (20 μ g), 3-OST-1 (10 μ g), and 35 S-labeled PAPS (30 μ M, 285,000 cpm/nmole) at 37°C for 2 hr. Alternatively, the oligosaccharides (1 μ g) or full-length *N*-sulfo heparosan (1 μ g) were preincubated with epimerase (8 μ g) at 37°C for 30 min, followed by incubation with a mixture of 6-OST-1 (20 μ g), 6-OST-3 (20 μ g), 2-OST-1 (40 μ g), and 3-OST-1 (10 μ g) in the presence of 35 S-labeled PAPS. The resultant 35 S-labeled oligosaccharides were incubated with AT, and the complex of AT and 35 S-labeled oligosaccharides was captured by a ConA-agarose column. The amount of 35 S-labeled products with full-length *N*-sulfo heparosan was defined to be 100%.

proliferation, suggesting that we have successfully separated the anticoagulant activity and cell proliferation activity by removing IdoUA2S and IdoUA residues from the polysaccharide.

The excellent activity and less structural heterogeneity of compound **2** led us to conduct the synthesis on a larger scale. We repeated the synthesis of **2** from deacetylated heparosan by using sequential modifications with *N*-sulfo-transferase (NST), 6-OST-1, 6-OST-3, and 3-OST-1 as described in Figure S2. In this synthesis, we employed a 3'-phosphoadenosine 5'-phosphosulfate (PAPS) regeneration system that used *p*-nitrophenol sulfate (PNPS) as a sulfo donor in place of PAPS; this substitution potentially reduces the cost of the synthesis by more than 1000-fold [7, 25]. Disaccharide analysis on the intermediate compounds, including *N*-sulfo heparosan and *N*-sulfo heparosan 6-*O*-sulfate, revealed that *N*-sulfation reached 75.6% and that 6-*O*-sulfation reached 85% (Table S1). The 3-*O*-sulfation carried out by 3-OST-1 went to about 90% completion, and it was estimated to be 0.5 3-*O*-sulfo groups/disaccharide based on the modification with 35 S-labeled PAPS. The final product, renamed recomparin (for recombinant heparin), showed anti-Xa activity at the IC₅₀ value of 25 ng/ml, which is very close to the value of heparin (19 ng/ml) (Figure S3). Taken together, our results show that the enzyme-based approach is fully capable of synthesizing milligram amounts of this anticoagulant polysaccharide.

In summary, we report a novel, to our knowledge, approach to prepare HS polysaccharides with different sul-

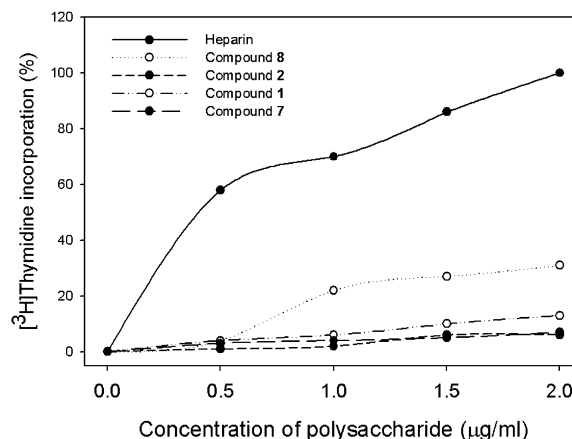


Figure 4. The Effect of the Synthetic Polysaccharides on FGF2-Dependent BaF3 FGFR1c Cell Proliferation

BaF3 FGFR1c cells were seeded in 96-well plates with 2 nM FGF2 for control and 2 nM FGF2 plus various concentrations of synthetic polysaccharides and heparin. Cells were cultured for 40 hr, followed by incubation in the media containing [3 H]thymidine for 4 hr. The cellular proliferation was determined by [3 H]thymidine incorporation into the DNA.

fation patterns. The compounds are used to investigate the contribution of the distribution of the sulfo groups to the biological functions. Utilizing the *in vitro* HS biosynthetic system, we can prepare a variety of HS structures by including or excluding certain enzymes. Unlike the chemical sulfonation approach, our method permits the synthesis of polysaccharides that are restricted by certain types of sulfations due to the high substrate specificities of the sulfotransferases. More importantly, this approach permits the synthesis of biologically active polysaccharides that may mimic the action of HS under physiological conditions. Using this approach, we have found that anticoagulant polysaccharides do not require the IdoUA residue. The characterization of the precise structure of the anticoagulant without IdoUA is currently underway. We hope that this structure will aid in the simplification of the synthesis of anticoagulant drug with reduced untoward effects. Due to the versatility and flexibility of the enzymatic synthesis, this approach will become an increasingly important tool for identifying the lead structures for the development of HS-based therapeutic agents.

SIGNIFICANCE

Heparan sulfate (HS) is a major component on the mammalian cell surface and in the extracellular matrix and has a wide range of biological functions. There is considerable interest in developing HS-based drugs to inhibit tumor growth, bacterial viral infections, and bacterial infections and to modulate inflammatory responses. It is clear that an enzymatic approach is a viable method for generating the polysaccharides with different sulfation patterns and biological functions. The synthesis can be conducted in parallel to

produce a large number of compounds by varying the levels of individual enzymes. The next challenge for HS/heparin research is the development of a technique to prepare “recombinant polysaccharides” with more precise structures so as to achieve high functional selectivity. In the coming years of the glycomics era, continuing efforts to improve the enzymatic synthesis of HS will lead to the development of novel therapeutic reagents.

EXPERIMENTAL PROCEDURES

Expression of HS Biosynthetic Enzymes

Expression of HS biosynthetic enzymes, including NST, epimerase, 2-OST, 6-OST-1, and 3-OST-1, was carried out in *E. coli* as described previously [7, 26–28]. The expression of 6-OST-3 was also carried out in *E. coli*. Briefly, the catalytic domain of mouse 6-OST-3 (Pro¹²¹–Pro⁴⁵⁰) was cloned into a pMalc2x vector (New England BioLab) from a mouse brain Quick clone cDNA library (BD Biosciences). The expression was carried out in Origami-B cells (Novagen) carrying pGro7 (Takara, Japan) plasmid expressing chaperonin proteins GroEL and GroES of *E. coli*. Transformed cells were grown in 6 l LB medium supplemented with 2 mg/ml glucose, 12.5 µg/ml tetracycline, 15 µg/ml kanamycin, 35 µg/ml chloramphenicol, and 50 µg/ml carbenicillin at 37°C. When the A₆₀₀ reached 0.4–0.7, isopropyl-thiogalactopyranoside (0.15 mM) and L-arabinose (1 mg/ml) were added to induce the expression of 6-OST-3 and chaperonin proteins, respectively. The cells were allowed to shake overnight at 22°C. Purification was carried out with an amylose-agarose (New England BioLab) column by following the protocols provided by manufacturer.

Preparation of Enzymatically Modified Polysaccharides

N-sulfo heparosan was prepared by incubating chemically deacetylated heparosan with purified NST and the components of the PAPS regeneration system described below. N-sulfo heparosan (150 µg) was incubated with ³⁵S-labeled PAPS (120 µM, 4.5 × 10³ cpm/nmole) and the combination of epimerase (2.7 mg if included), 2-OST (8 mg if included), 6-OST-1 (1.2 mg if included), 6-OST-3 (1.4 mg if included), and 3-OST-1 (0.9 mg if included) in 50 mM MES and 1% Triton X-100 (pH 7.0). The reactions were incubated at 37°C for 2 hr with shaking. The resultant ³⁵S-labeled polysaccharides were purified by DEAE chromatography [7].

To prepare the enzymatically modified oligosaccharides, the N-³⁵S-labeled sulfo oligosaccharide substrates (1 µg, 1,500 cpm) were incubated with a mixture of 6-OST-1 (20 µg), 6-OST-3 (20 µg), 3-OST-1 (10 µg), and ³⁵S-labeled PAPS (50 µM, 2.85 × 10⁵ cpm/nmole) in 100 µl 50 mM MES and 1% Triton X-100. Alternatively, the oligosaccharide substrates (1 µg) were incubated with epimerase (7 µg) at 37°C for 30 min before they were incubated with a mixture of 2-OST (40 µg), 6-OST-1 (20 µg), 6-OST-3 (20 µg), and 3-OST-1 (10 µg). The reactions were terminated by heating at 100°C for 2 min. The resultant oligosaccharides were mixed with AT to determine binding by using a concanavalin A (ConA)-agarose column as described below.

Disaccharide Analysis of Polysaccharides

The ³⁵S-labeled HS was degraded with nitrous acid at pH 1.5, followed by reduction with sodium borohydride [29]. The resultant ³⁵S-labeled disaccharides were resolved by a C₁₈ reverse-phase column (0.46 × 25 cm) (Vydac) under reverse-phase ion pairing HPLC conditions. The identities of the disaccharides were determined by coeluting with appropriate ³⁵S-labeled disaccharide standards [30].

Determination of the Amount of Polysaccharides

The amount of the synthetic polysaccharides was estimated by a colorimetric method with alcian as described [31]; in this method, the standard curve was generated by using HS isolated from bovine

kidney. To measure the concentrations of heparosan, N-sulfo heparosan, and N-sulfo heparosan 6-O-sulfate, the polysaccharides were depolymerized to disaccharides by using a mixture of heparin lyases I, II, and III. The amount of the resultant disaccharides was determined by coeluting with disaccharide standards (from Seikagaku) on RPIP-HPLC as described above, by using UV 232 nm detection.

Antithrombin Binding

Approximately 1 × 10⁵ cpm ³⁵S-labeled compound was incubated with 5 µg human AT (Cutter Biological) in 50 µl binding buffer containing 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Mn²⁺, 1 mM Mg²⁺, 1 mM Ca²⁺, 10 µM dextran sulfate, 0.0004% Triton X-100, and 0.02% sodium azide for 30 min at room temperature. ConA-Sepharose (Sigma, 50 µl 1:1 slurry) was then added, and the reaction was shaken at room temperature for 1 hr. The beads were then washed three times in 1 ml binding buffer, and the bound polysaccharide was eluted with 1 ml 1 M NaCl.

Anti-Xa and Anti-IIa Assays

Assays were based on two previously described methods [32, 33]. Briefly, factor Xa (Enzyme Research Laboratories, South Bend, IN) and thrombin (Sigma) were diluted to 1 U/ml and 8 U/ml, respectively, with PBS containing 1 mg/ml BSA. AT was diluted with PBS containing 1 mg/ml BSA to give a stock solution at the concentration of 27 µM. The chromogenic substrates S-2765 (for factor Xa assay) and S-2238 (for thrombin assay) were purchased from Diapharma and prepared at 1 mM with PBS. The synthesized polysaccharides or heparin was dissolved in a buffer containing 50 mM Tris-HCl (pH 8.4), 7.5 mM Na₂EDTA, and 175 mM NaCl at various concentrations (1–10,000 ng/ml). The reaction mixture, which consisted of 25 µl AT stock solution and 25 µl of the solution containing polysaccharide, was incubated at 37°C for 2 min. Factor Xa (25 µl) or thrombin (25 µl) was added. After incubating at 37°C for 4 min, 25 µl S-2765 or S-2238 was added. The absorbance of the reaction mixture was measured at 405 nm continuously for 10 min. The absorbance values were plotted against the reaction time. The initial reaction rates as a function of concentration were used to calculate the IC₅₀ values.

Affinity Coelectrophoresis

The dissociation constant (K_D) of each sample and AT was determined by using affinity coelectrophoresis [34]. Approximately 4000–5000 cpm antithrombin-binding, ³⁵S-labeled polysaccharide was loaded per lane; there were zones of AT at concentrations of 0, 8, 16, 60, and 120 nM. The gel was run at 400 mA for 2 hr, dried, and then analyzed on a PhosphorImager (Amersham Biosciences, Storm 860). The retardation coefficient was calculated at R = (M₀ – M)/M, where M₀ is the mobility of the polysaccharide through the zone without AT, and M is the mobility of the polysaccharide through each separation zone. The retardation coefficient was then plotted against the retardation coefficient divided by its respective concentration of AT. The slope of the line represents –1/K_D.

FGF2/FGFR1c-Mediated Proliferation Assay

The BaF3 cells ectopically expressing FGFR1c have been described previously [24]. The BaF3 FGFR1c cells were maintained in RPMI 1640 media (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum, 0.5 ng/ml IL-3 (PeproTech, Inc., Rocky Hill, NJ), 2 mM L-glutamine, penicillin (50 IU/ml), and streptomycin (50 µg/ml), and 50 µM β-mercaptoethanol. For mitogenic assays, BaF3 FGFR1c cells were washed three times with RPMI 1640 media to remove IL-3 and resuspended in the growth media lacking IL-3. About 30,000 cells were plated per well in a 96-well plate in media containing various concentrations of heparin, compounds 1, 2, 7, and 8, and 2 nM FGF2 (PeproTech, Inc.) in a total volume of 200 µl. The cells were then incubated at 37°C for 40 hr. To each well, an additional 50 µl growth media containing 1 µCi [³H]thymidine was added. Cells were harvested after 4–5 hr by centrifugation. The incorporation of [³H]thymidine into the DNA was determined by scintillation counting.

PAPS Regeneration System

The reactions involved in the PAPS regeneration system are shown in Figure S2. N-terminal His₆-tagged AST-IV was expressed in *E. coli* and purified as described by Burkat and colleagues [25] at a yield \approx 50 mg/l bacterial culture.

Preparation of N-Sulfo Heparosan

Heparosan was isolated from *E. coli* K5 strain by using a DEAE column [35]. The deacetylated heparosan was achieved under alkaline conditions [36]. The deacetylated heparosan was incubated with purified NST and coupled with a PAPS regeneration system. Briefly, 2.5 mg purified AST-IV was incubated with 40 μ M PAP and 1 mM *p*-nitrophenol sulfate (PNPS) in 40 ml 50 mM MES (pH 7.0), 1% Triton X-100, 1 mM MgCl₂, and 1 mM MnCl₂ at 25°C for 15 min. The reaction mixture was mixed with *N*-sulfotransferase (10 mg), 5 mg deacetylated heparosan was added, and the mixture was rotated at 25°C for 24 hr. The *N*-sulfo heparosan was recovered by a DEAE column. The resultant product was subjected to a mixture of heparin lyases, followed by a disaccharide analysis to assess the level of *N*-sulfation. After two rounds of the modifications, the *N*-sulfation level reached 80%.

Preparation of N-Sulfo Oligosaccharides

N-³⁵S-labeled sulfo heparosan (1 mg, 1.5×10^6 cpm) was mixed with 20 ng purified heparin lyase III in 1 ml 50 mM sodium phosphate (pH 7.0). The digestion lasted for 18 hr and was terminated by heating at 100°C for 2 min. The digested polysaccharide was resolved on a Bio-Gel P-10, which was eluted with a buffer containing 25 mM Tris and 1000 mM NaCl (pH 7.4) at a flow rate of 2 ml/hr.

Supplemental Data

Supplemental Data include the preparation and characterization of recomparin and are available at <http://www.chembiol.com/cgi/content/full/14/9/986/DC1/>.

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